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## Note

# Simple device for high-performance liquid chromatographic separation on microbore columns

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High-performance liquid chromatography (HPLC) has played a very significant role in isolation, purification and structural studies and more so for compounds of biological importance, such as proteins, peptides and amino acids. The technological advancement of HPLC has paralleled the need for analyzing compounds available in low amounts. One such advancement is the miniaturization of HPLC columns to achieve higher sensitivity. The most commonly employed columns in the past have been 4–30 cm long with internal diameter (I.D.) of 0.4 to 0.6 cm. In recent years[1], however, there has been a considerable interest in columns of smaller diameter (1–2 mm I.D.), commonly known as "microbore" columns. The efficiency of these columns is generally higher, thus improving the separation of complex mixtures or hard to resolve molecules. Although various factors influence the effeciency of microbore columns, the pumping system has often been the limiting factor, particularly if the separation is achieved by gradient mode. For microbore column chromatography the most desired aspects of HPLC are its capability of (i) pumping mobile phase at a slower flow-rate (50–200 μl/min), (ii) generating accurate gradients at low flow-rates and (iii) spectrophotometric detection in small volumes of eluent. Commonly available HPLC systems are not designed to meet these requirements with reasonable precision and accuracy. In this communication, a very simple and economical device is described to modify a conventional HPLC system (single- or dual-pump) for microbore column chromatography. The modification requires minimum plumbing, can be used with or without autoinjector, and can be installed within a short period of time. The device has been used extensively in this laboratory in conjunction with protein studies, and the results are presented in this communication.

## MATERIALS AND METHODS

### *Chemicals*

Chemicals for HPLC were purchased from EM Science (Cherry Hill, NJ U.S.A.) and for amino acid sequence anlysis from Spinco Division of Beckman Instruments (Palo Alto, CA, U.S.A.), and Pierce (Rockford, IL, U.S.A.).

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*Instrumentation*

Sequence analyses were performed on a Beckman 890M sequencer as described earlier[2], and phenylthiohydantoin (PTH) derivatives of the cleaved amino acids were identified by HPLC with a macrobore (4.5 mm I.D.) column as described previously[3] and with a microbore (2.0 mm I.D.) column as described in this communication. To examine the efficiency of the device three different HPLC systems were employed: Perkin-Elmer Series 4 microprocessor-controlled solvent delivery system (single-pump); Waters HPLC (dual-pump) equipped with two 6000A solvent delivery pumps and a System controller (Model 720); Beckman HPLC (dual-pump) equipped with a 421A controller, two 110B solvent delivery modules and a system organizer. All three HPLC systems were equipped with Waters autoinjector WISP 710B and a dual-channel absorbance detector containing a micro cell (1-$\mu$l capacity, Waters Assoc., Cat. No. 97379).

Ultrasphere 5-$\mu$m ODS macrobore and microbore columns (150 × 4.6 mm I.D. and 150 × 2.0 mm I.D., respectively) manufactured by Altex and marketed by Beckman Instruments (parts No. 235330 and 237390) were employed to separate the PTH derivatives.

*Modification*

In order to adopt the conventional HPLC system to microbore column chromatography the solvent delivery systems were modified, as shown in Figs. 1 and 2. Irrespective of the HPLC system used (one pump or two pumps) the tube from the mixing chamber/solvent delivery system was connected to a "T" ("C" in Fig. 1 and 2) with a minimum dead volume. One outlet of the "T" was connected in series to a fine metering valve (Cat. No. 55-22RF2; "D" in Fig. 1 and 2) manufactured by Whitey Co. (Highland Hts., OH, U.S.A.) and to a pulse damper (Water Assoc., Millford, MA, U.S.A.; Cat. No. 98060; "E" in Fig. 1 and 2). The second outlet from the "T" joint was connected to the autoinjector ("F" in Fig. 2) through a stainless-steel tube of minimum length and 0.007 in. I.D. The solvent delivery tube (0.007 in. I.D.) from the injector to the column ("G" in Fig. 2) and from column to the detector

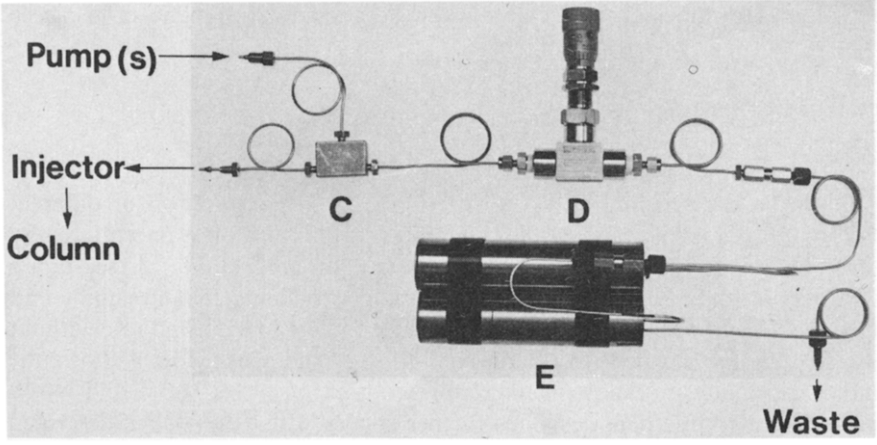


Fig. 1. Arrangement of "T", fine metering valve and pulse damper, C = "T"; D = fine metering valve; E = pulse damper.

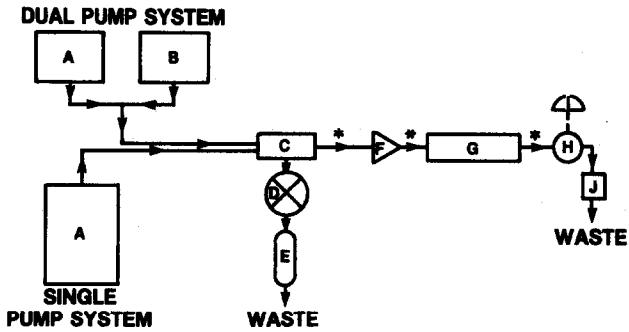


Fig. 2. Schematics of the plumbing for fine metering valve in one pump or two pumps conventional HPLC. A and B = HPLC pumps; C = "T"; D = fine metering valve; E = pulse damper; F = autoinjector; G = microbore column; H = detector; I = recorder; J = back pressure restrictor; * = 0.007 in I.D. stainles-steel tubing.

("H" in Fig. 2) was also of shortest possible length. Tube length and 0.007 in. I.D. for plumbing is critical. Because of the slow flow-rate and small volume (1.0 $\mu$l) detector cell (Waters Assoc.; Cat. N. 97379) it was necessary to create a back pressure inside the detector cell to avoid the possibility of air bubble formation. This was achieved by introducing a back pressure restrictor (manufactured by Upchurch, Oak Harbor, WA, U.S.A.; Cat. No. U4462; "J" in Fig. 2) at the outlet side of the detector cell ("H" in Fig. 2). The only plumbing change in the autoinjector was to disconnect the 2.0-ml loop of the injector assembly.

Separation of standard PTH-amino acids and residue from the sequencer run was achieved by the procedure described previously[3]. The flow-rate was 1.5 ml/min for both macrobore and microbore columns; however, for the microbore column the metering valve was adjusted to maintain the column back pressure (which can be read on the controller screen) at the same level as with the microbore system (*ca.* 2000 p.s.i.). This corresponds to flow of 0.320 $\mu$l/min through the macrobore column. The excess solvent was directed to the waste through the "T" joint and pulse damper. Injection volumes for the microbore column were kept to a minimum, never to exceed 25 $\mu$l.

## RESULTS AND DISCUSSION

Fig. 1 shows the arrangement of "T" (C), fine metering valve (D) and pulse damper (E) while the schematics is shown in Fig. 2. The separation of different amounts (50–100 pmol) of standard PTH amino acid mixture on a microbore and macrobore column at two different sensitivities is compared in Fig. 3 (see figure legends for details). It is evident from Fig. 3 that by introducing split streaming and metering valve (Figs. 1 and 2) it is possible to reduce to flow to a desired rate without adversely effecting the gradient to obtain resolution of the peaks. Fig. 4 shows the PTH-amino acid analysis results on microbore column of cycles No. 5 and No. 9 from two different tryptic peptides. This further suggests that the system described here can be effectively used for the analysis of the samples obtained from the amino acid sequencer run.

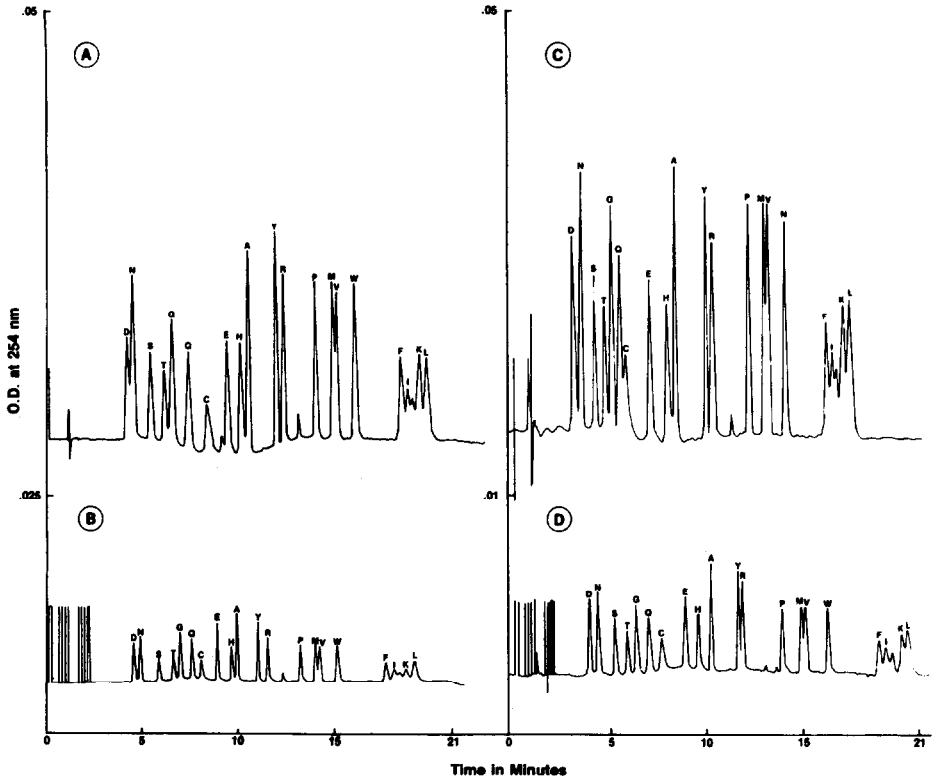


Fig. 3. Separation of standard PTH-amino acid mixture. (A) 100 pmol on microbore column using dual-pump system; (B) same as in A on macrobore column; (C) 50 pmol on microbore column using single-pump system; D = same as in C on macrobore column. Single-letter symbols are used for amino acids.

Most of the available HPLC units are capable of performing gradient elution on macrobore columns; however, they are not at their best performance at a lower flow-rate, which is a prerequisite for gradient elution on microbore columns. In order to surmount this problem a number of approaches have been taken[4–8]. One such approach has been flow splitting, described by Van der Wal and Yang[5] on a single-pump system without the use of autoinjector. Moreover, they have reported a gradient system for fused-silica columns and not a high-pressure system. Alternatively a gradient storage procedure has been described by a number of investigators[6–7], and more recently by Schachterle and Alfredson[9]. This procedure, although it allows gradients to be run at slower flow-rates, needs sophisticated programming because of the system hydraulics. Furthermore, efficiency of both these systems has not been described in conjunction with autoinjector and/or dual-pump system. An autoinjector is essential when continuous unattended runs are performed, particularly in the structural studies of proteins and in industrial laboratories.

The split stream system described in this paper gives reproducible results and can be adapted to a single- or dual-pump conventional HPLC system with or without the autoinjector. In addition, by closing the metering valve completely, macrobore

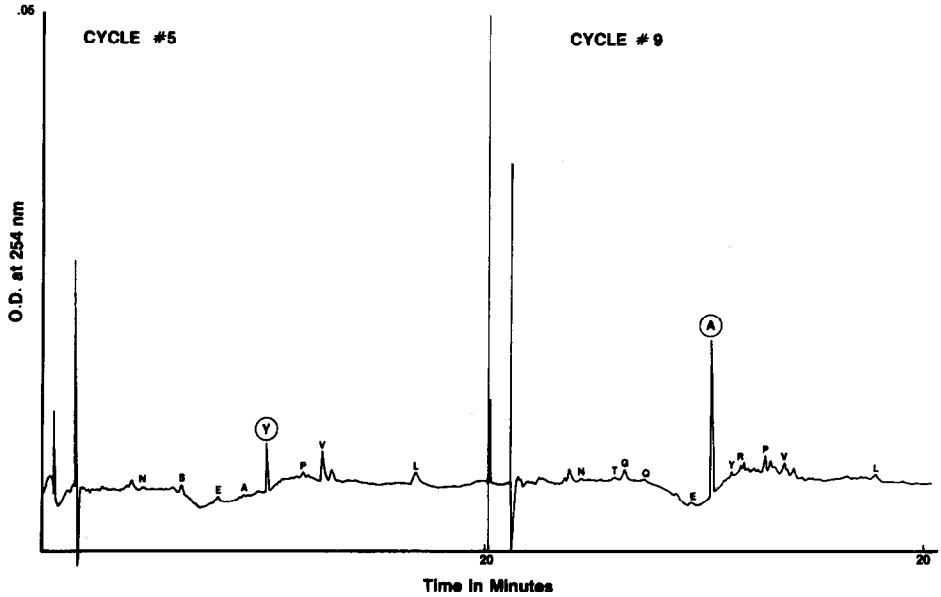


Fig. 4. PTH-amino acid analysis results on microbore column from two different tryptic peptides. Actual residue is circled. Single-letters symbols are used for amino acids.

column HPLC can be performed, thus avoiding any plumbing changes. It is interesting to note that since the separation is achieved at a pressure equivalent to that used with the macrobore column, the gradient forming program is the same for both types of column, as evident from Fig. 3. Because of the fine metering valve, virtually any flow-rate can be achieved, without any major plumbing changes. Currently we are in the process of automation of the metering valve, by linking it to the HPLC controller to read the pressure values through an added microprocessor, and thus regulate the metering valve to keep the flow constant during the run.

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